

Immunoglobulin-bearing lymphoid cells in primary syphilis

Quantitative and elution studies

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SUMMARY The delay in antibody production in response to infection with *Treponema pallidum* may be caused by a block in the differentiation of antigen-stimulated B (Bursa-dependent) lymphoid cells towards plasma cells. This hypothesis was tested by a study to detect clonal expansion of immunoglobulin-bearing B lymphoid cells by in-vitro immunofluorescence tests in patients with primary syphilis. In addition, antibodies eluted from circulating lymphoid cells were investigated for treponemal binding by the enzyme-linked immunosorbent assay, the *T pallidum* immobilisation test, and the immunoglobulin class-specific FTA-ABS test.

Results indicated that the number of IgG-bearing lymphoid cells were increased in patients with primary syphilis. However, in only a few cases could antitreponemal antibodies be eluted from isolated lymphoid cells. For this reason, the original hypothesis was rejected.

Introduction

In syphilis the immunological response of the host to infection with *Treponema pallidum* has been extensively studied. As far as the cellular limb of immunity is concerned, many findings have pointed to impaired cell-mediated immunity in the early stages of infection.¹ Studies of the humoral response have shown that after infection immunoglobulins of different classes (IgG, IgA, IgM, and IgE) are extensively produced and their raised concentrations in serum and (at least partial) antitreponemal specificity have been demonstrated.²⁻⁶

Although *T pallidum* is present in the body after inoculation, there is often a delay before specific immunoglobulins can be detected. A temporary delay or block in the differentiation of antigen-stimulated B lymphoid cells into immunoglobulin-secreting plasma cells may be the origin of this delay.

The aim of the present study was to investigate this hypothesis. It was based on the previous findings of Cormane *et al*⁷⁻⁹ that disease-specific antibodies could be eluted from circulating lymphoid cells in patients with autoimmune diseases such as lupus erythematosus, pemphigus, pemphigoid, scleroderma, and alopecia areata.

In this study the antitreponemal specificity of antibodies eluted from circulating lymphoid cells from patients with primary syphilis were investigated by the immunoglobulin-class-specific fluorescent treponemal antibody absorption (FTA-ABS) test, the *T pallidum* immobilisation (TPI) test, and the enzyme-linked immunosorbent assay (ELISA). At the same time, the number of immunoglobulin-bearing (B lymphoid) cells was estimated for each of the immunoglobulin subclasses by in-vitro immunofluorescence studies.¹⁰

Patients and methods

STUDY GROUP

The patients included in this study were referred from the outpatient department of our hospital. All were male, and a diagnosis of primary syphilis was based on the existence of an indurated ulcer from which *T pallidum* could be demonstrated by dark-field microscopy and a positive result to a treponemal serological test at the initial or at a subsequent examination.

A total of 12 patients was studied; of these, 10 were untreated at the time of investigation and two had been treated for less than one week. The time interval between infection and time of study varied from four to 12 weeks.

A complete study of sera and lymphoid cell eluates from 10 patients was carried out (Table I). In addition, B lymphoid cell subpopulations were quantitatively determined in 10 patients (Table II).

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TABLE I Results of serological tests performed on sera of 10 patients with primary syphilis

Case No	Serological test results					FTA-ABS test using				
	Kolmer	VDRL	RPCF	FTA-ABS	TPI	IgG	IgA	IgM	IgD	IgE
1	-	+/-	+	+	-	+	-	+	-	+
2	-	-	-	+/-	-	+	+	+	-	+/-
3	-	+/-	-	+/-	-	+/-	-	+	-	-
4	+	+	+	+	+	+	-	-	-	+
5	-	+/-	-	+/-	-	+/-	-	-	-	+
6	+	+	+	+	+	+/-	-	+	-	+
7	-	-	-	+	-	+/-	-	-	-	-
8	+	+	+	+	+	+/-	-	+	-	+
9	+	+	-	+/-	-	+	-	+	-	+
10	-	+/-	+	+/-	-	-	-	+	-	-

+ Positive - negative +/- doubtful positive

TABLE II Percentage of peripheral blood lymphocytes bearing surface immunoglobulin in 10 patients with primary syphilis

Case No	Time after infection (weeks)	Percentage bearing immunoglobulins				
		IgG	IgA	IgM	IgD	IgE
1	7	9	0	5	3	2
2	6	25	2	15	5	4
4	6	11	1	5	4	1
5	NS	15	0	5	1	2
6	NS	25	2	10	10	7
7	4	7	3	2	1	2
8	4	4	1	2	2	2
9*	8	1	2	3	0	2
11	NS	25	2	8	6	11
12*	12	12	2	3	1	2
Range	4-12	1-25	0-3	2-15	0-10	1-11
Median		11.5	2.0	5.0	2.5	2.0
Standardised W test		2.6537	0.7177	0.0000	-0.6070	1.3384
P		0.0089	0.4979	1.0304	0.5693	0.1943

NS=Not specified

*Patients treated for less than one week

CONTROLS

Ten healthy staff members served as controls for the quantitative determination of B lymphoid cells. All were male and of the same age range as the study group.

SEROLOGICAL TESTS

The FTA-ABS test* was performed according to the standard method described by Kawamura *et al.*¹¹ It was modified as described before.⁶

The Treponematoses Department of the National Institute of Public Health (NIPH) investigated all sera with the Kolmer modification of the Wassermann reaction, the Venereal Disease Research Laboratory (VDRL) test, the Reiter protein complement fixation (RPCF) test, the FTA-ABS

test, and the TPI test. Lymphoid cell eluates were investigated with the TPI and ELISA tests.

ISOLATION OF LYMPHOID CELLS

Lymphoid cell separation was carried out according to the method of Böyum,¹² with some minor modifications as described below.

Fifty millilitres of peripheral blood was collected by venepuncture in Erlenmeyer flasks containing 50 glass beads and shaken for 10 minutes for defibrination. To this, 70 ml of phosphate-buffered saline (PBS), pH 7.4, was added. Portions of 40 ml of this suspension were layered on top of portions of 10 ml Ficoll-Isopaque mixture. The suspensions were centrifuged at $400 \times g$ for 20 minutes. The resulting lymphoid-cell-rich layer between Ficoll-Isopaque and supernatant was aspirated and washed three times in PBS. After each washing, the lymphoid cell suspension was centrifuged at $200 \times g$ for five

* Specifications of the antisera used may be obtained from the authors.

minutes. The cell layer obtained was composed of 90-95% lymphoid cells and 5-10% monocytes and neutrophils. Viability of the isolated cells was over 98% as determined by trypan blue exclusion. The last washing solution served as control for subsequent immunoglobulin studies.

ELUTION OF IMMUNOGLOBULINS

The lymphoid cells were resuspended in 0.5 ml of a 0.05 mol/l citrate buffer (pH 3.2) and incubated overnight at 4°C. After incubation, the suspension was centrifuged at $200 \times g$ for 10 minutes and the supernatant (now called eluate) brought to a pH of 7.2 by addition of 0.1 mol/l NaOH.

Without dialysis or concentration the eluate was tested for the presence of antitreponemal immunoglobulins: (a) undiluted with class-specific FTA antisera; (b) diluted 1/2 in sorbent with class-specific FTA antisera; (c) diluted 1/12 by the TPI test; and (d) diluted 1/10 and 1/100 by ELISA.

IMMUNOFLUORESCENCE STUDIES

Fluorescein isothiocyanate (FITC)-labelled antisera directed against human IgG, IgA, IgM, IgD, and IgE were obtained from Meloy Laboratories (Springfield, Illinois).*

Direct staining of isolated lymphoid cells in suspension was carried out by suspending 0.1 ml washed viable cells (2×10^5) in 0.1 ml of each conjugate (final conjugate dilution 1/40). This mixture was then incubated at room temperature for 30 minutes. After incubation the cells were washed twice for five minutes at $200 \times g$ in PBS and resuspended in 0.2 ml PBS.

In each case, the percentage of IgG-, IgA-, IgM-, IgD-, and IgE-positive cells was determined by examining the microscopic field alternately in blue narrow-band epi-illumination (480-490 nm) for fluorescence and in conventional light.

FLUORESCENT MICROSCOPY

A Leitz Orthoplan microscope with a Xenon XBO-75 lamp and a Leitz vertical illuminator was used. The primary filter combination was BG 38 (4 mm), KP 490 (2 mm), GG 475 (2 mm), and the secondary filter was an OG 513 (3 mm). For routine use, examination was performed with a $\times 10$ ocular and a Leitz $\times 40$ dry objective.

PERIPHERAL BLOOD COUNTS

Total peripheral leucocyte counts and their differentiation were estimated to relate percentage figures to the absolute numbers of lymphoid cells.

STATISTICAL METHOD

The non-parametric Wilcoxon's test,¹³ with the usual correction for ties and the normal approximation to obtain the P value, was used for statistical analysis of the numbers of immunoglobulin-bearing lymphoid cells in patients with primary syphilis and in healthy controls.

TEST MATERIALS

Isopaque (Natrii N-, ethyl-3,5-diacetamido-2.4.6.-trijodbenzoas) solution: 20 ml Isopaque (Nyegaard and Co, Oslo, Norway) was diluted with 25 ml distilled water.

Isopaque-Ficoll mixture: 10 parts Isopaque solution were mixed with 24 parts of Ficoll (9%) (Pharmacia, Uppsala, Sweden).

Citrate buffer, pH 3.2, 0.05 mol/l: 43.7 ml 0.1 mol/l citric acid + 6.3 ml 0.1 mol/l sodium citrate diluted with demineralised water to 100 ml.

0.1 mol/l citric acid: 21.02 g citric acid monohydrate diluted with demineralised water to 1 litre.

0.1 mol/l sodium citrate: the dihydrate rather than the pentahydrate should be used: 29.4 g of trisodium citrate dihydrate dissolved in CO₂-free water, and the volume made up to 1 litre.

pH indicator paper pH 6.4-8.0 was obtained from E Merck, Darmstadt, W Germany.

Trypan blue: 0.05% in PBS.

Results

SEROLOGICAL TESTS

Results of serological tests on the sera of cases 1-10, performed in the NIPH and in our own laboratory, are given in Table I. Since it was the aim of this study to investigate patients in the earliest stage of primary syphilis, all results are presented. From the results of tests performed by the reference laboratory it may be concluded that three patients (cases 2, 3, and 5) were sero-negative at the time of investigation as they had only doubtful VDRL or FTA-ABS test results or both. However, two patients (cases 2 and 3) had positive results to the IgM-FTA-ABS test in our own laboratory, which may not have been detected by the anti-Ig (anti-total) conjugate used in the NIPH. Only two patients (cases 4 and 8) were unequivocally sero-positive.

B LYMPHOID CELL DETERMINATION

The results of B lymphoid cell determination in 10 patients with primary syphilis are given in Table II and those of 10 healthy controls in Table III. Absolute numbers of lymphoid cells in the study group did not differ from normal (mean: $2.35 \times 10^9/l$; range $1.66-2.95 \times 10^9/l$), and statistical analysis was performed on percentage figures.

* Specifications and working dilutions of antisera used for immunofluorescence studies may be obtained from the authors.

TABLE III Percentage of peripheral blood lymphocytes bearing surface immunoglobulins in 10 healthy controls

Control No	Percentage bearing immunoglobulins				
	IgG	IgA	IgM	IgD	IgE
1	2	0	8	6	2
2	2	0.5	1	1.5	1
3	7	2	8	8	3
4	3	1	6	4	5
5	3	0.5	3	3.5	1.5
6	2.5	2	6	3	1
7	3.5	1.5	3	1.5	2
8	4	1	2	2.5	2
9	8	2	7	2	1
10	6	2	6	2.5	2
Range	2.8	0.2	1.8	1.5-8	1.5
Median	3.25	1.2	5.0	3.5	2

The exact values of standardised W and two-sided tail probability, obtained by applying Wilcoxon's test to each of the five investigated immunological traits, are given in Table II. The study group and the control group differed significantly in the number of IgG-bearing lymphoid cells ($P 0.0089$). The number of IgG-bearing lymphoid cells had increased in the study group, its median being 8.25% higher than that of the control group. No significant difference was found in the other subclasses of immunoglobulin-bearing lymphoid cells.

ELUTION STUDIES

In only two cases was a clearly positive IgM-FTA test result obtained with undiluted eluates of isolated lymphoid cells. Of these, one patient (case 1) continued to have a positive result, and the result of another (case 8) became negative after 1/2 dilution of eluate in sorbent (IgM-FTA-ABS test). Borderline FTA test results (undiluted) were obtained twice for IgG (cases 2 and 8), once for IgM (case 2), and once for IgD (case 3). The results of the IgA-FTA and IgE-FTA tests, ELISA, and TPI test were all negative as were the findings in the last washing solutions.

Discussion

The results of determining the numbers of immunoglobulin-bearing lymphoid cells in primary syphilis indicate that the number of IgG-bearing lymphoid cells are significantly increased, with a probability of $P = 0.0089$.

In the study group, circulating antitreponemal IgM antibodies were detected in five out of eight patients, the remaining three probably having false-negative results. The production of circulating antitreponemal IgG seemed to be only beginning, as the results in four out of eight cases to the IgG-FTA-ABS test were positive and those in the other four were borderline (Tables I and II).

The initiation of antitreponemal IgG production—with intervals between the date of infection and the time of investigation varying from four to 12 weeks—and the possible clonal expansion of IgG-bearing lymphoid cells seemed to confirm that a block in the differentiation of antigen-stimulated B lymphoid cells towards secreting plasma cells might be responsible for the delay in detectable antibody production in early syphilis. However, the results of the serological tests (TPI, ELISA, class-specific FTA and FTA-ABS tests) made us reject that hypothesis. No group-specific or species-specific antitreponemal antibodies could be demonstrated consistently in the eluates, not even from increased numbers of circulating IgG-bearing lymphoid cells, indicating that these were not precursors for antitreponemal antibody-producing plasma cells.

Thus, the increased number of IgG-bearing lymphoid cells in primary syphilis, as detected by surface membrane Ig-marking, may have originated from another immunological phenomenon.¹⁰ Firstly, the technique may have detected IgG-treponemal antigen immune complexes bound via Fc receptors to non-B lymphoid cells.¹⁴ Secondly, anti-lymphocyte autoantibodies of the IgG-class may have been detected by the test procedure. Although these antibodies have not as yet been detected in syphilis, a T cell inhibitory factor is known to exist in syphilitic sera.^{1, 15}

The rejection of the original hypothesis, and thus the probability of normal B lymphoid cell differentiation in primary syphilis, is in agreement with the role of the extracellular material at the surface of *T pallidum*.^{16, 17} This layer may not only prevent non-immunological defence mechanisms from interfering with the invading micro-organism but may also cause a delay in antibody production as a consequence of its low antigenic capacity. Moreover, it may explain the limited success of in-vivo passive immunisation with syphilitic sera in rabbit experiments.^{18, 19} The finding of increased numbers of IgG-bearing lymphoid cells in patients with primary syphilis needs to be confirmed but it may be definitively explained by future investigations.

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